IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of	
Masao MATSUOKA et al.	Group Art Unit: 1636
Application No.: 10/667,359	Examiner: C. Qian
Filed: September 23, 2003	Confirmation No.: 9190
For: METHOD TO PROTECT TRANSGENES FROM SILENCING	

DECLARATION OF DR. SHOJI TAJIMA

Commissioner for Patents
P.O. Box 1450
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Sir:

1. I, Shoji TAJIMA, declare the following:

2. I am a citizen of Japan, and have the following mailing address: Institute for Protein Research, 3-2 Yamadaoka, Suita, Osaka 565-0871, Japan;

3. I graduated from Osaka University with a Ph.D. degree in 1978;

4. I am a professor in the Laboratory of Epigenetics at the Institute for Protein Research, Osaka University, Osaka, Japan, and have held this position since the year 2005;

5. I am collaborating with Dr. Masao Matsuoka on research activities related to the invention disclosed in the above-identified application, and I am submitting this Declaration in support of that application;

6. I have performed and/or supervised the experiments reported below;

7. In the experiments, the anti-silencing effects of sea urchin arylsulfatase insulator were measured using the experimental system that is described in Akasaka et al. (Akasaka et al., Cellular and Molecular Biology (1999) 45, 555-569). In particular, a reporter gene (green fluorescent protein gene: GFP gene) was introduced into Hela cells with or without sea urchin arylsulfatase insulator in a sense (+) or an anti-sense (-) orientation. Fig. 1, submitted herewith, shows the structures of the various reporter plasmids. As in Akasaka et al., and in contrast to the present invention, the reporter gene was introduced using a lipofectin system rather than a

viral vector. Fig. 2, also submitted herewith, illustrates the time-course of anti-silencing activity, represented by the fluorescence intensity of the various strains of transfected Hela cells. As shown in Fig. 2, the anti-silencing activity observed in experimental systems with sea urchin arylsulfatase insulator (Fig. 2B: sense orientation, Fig. 2C: antisense orientation) was not significantly different from the anti-silencing activity observed in control cells without insulator (Fig. 2A);

8. The results of these experiments demonstrate that the experimental system described in Akasaka et al. does not provide an anti-silencing effect in the presence of a sea urchin arylsulfatase insulator;

9. I further declare that all statements made herein of my own knowledge are true and that all statements on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful statements may jeopardize the validity of the application or any patent issuing thereon.

Date: June 4th, 2007 By: _____
Shoji TAJIMA

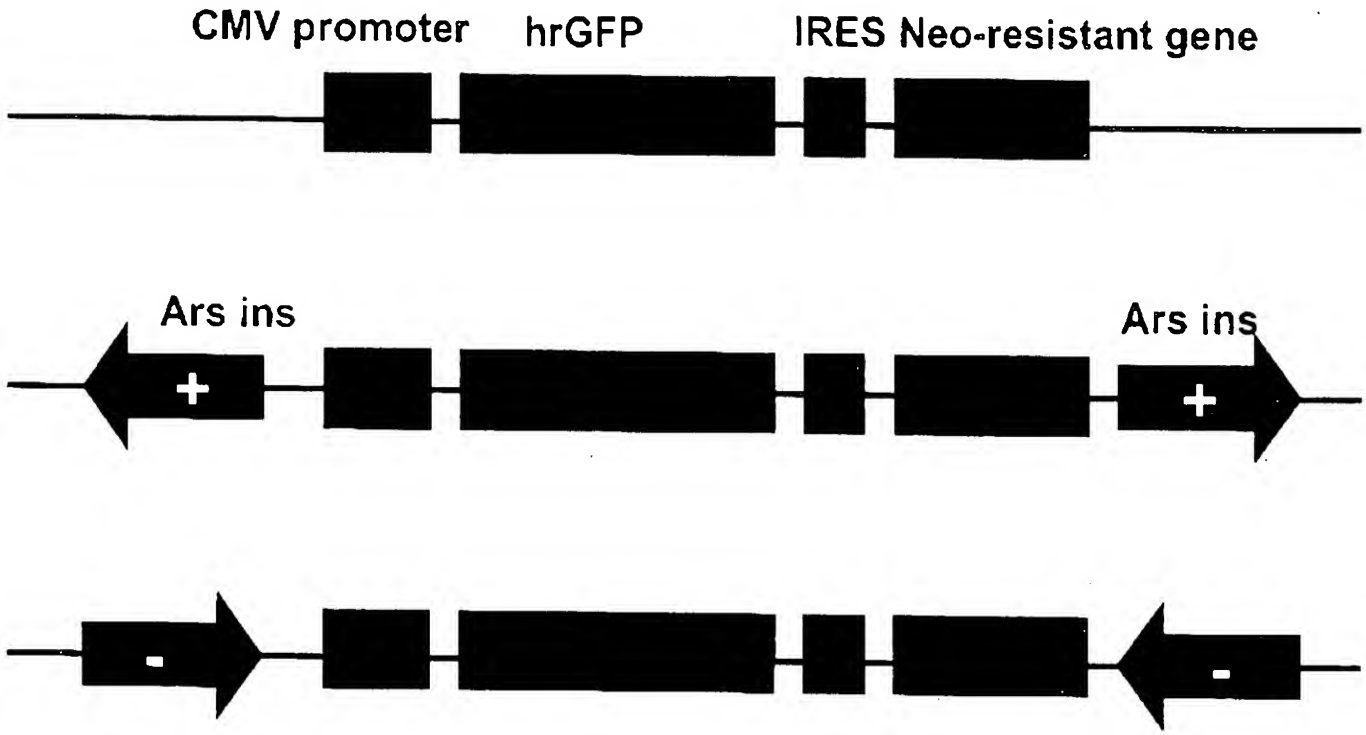

Figure 1. Structure of reporter plasmid DNA

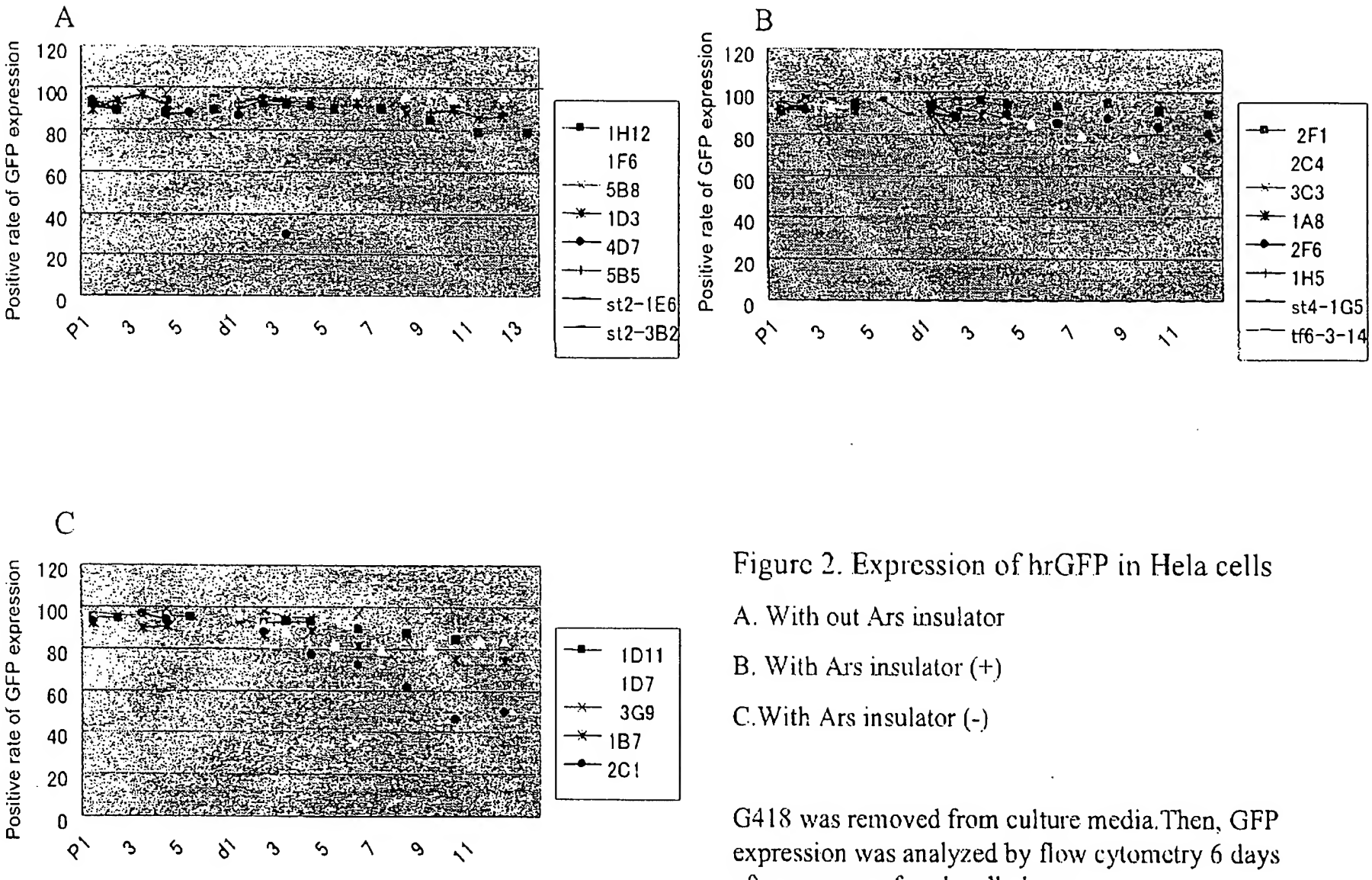

Figure 2. Expression of hrGFP in Hela cells

A. With out Ars insulator

B. With Ars insulator (+)

C.With Ars insulator (-)

G418 was removed from culture media.Then, GFP expression was analyzed by flow cytometry 6 days after passage of each cell clones.

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RESEARCH ARTICLE

Sea urchin insulator protects lentiviral vector from silencing by maintaining active chromatin structure

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Suppressed expression of transgenes in vivo is the major obstacle in the gene therapy. For the long-term expression, we utilized a chromatin insulator from sea urchin arylsulfatase (Ars) gene locus (Ars insulator, ArsI), which has been shown to epigenetically regulate gene expression across species. ArsI was able to prevent silencing of the transgene in a myeloid cell line, HL-60, and a murine embryonic stem cell line, CCE, in an orientation-dependent manner, but not in Huh-7, K562 and MCF-7 cells, indicating that the effect of ArsI on gene silencing was cell type dependent. Although anti-silencing effect of ArsI was almost equivalent to that of chicken β-globin insulator, incorporation of ArsI into lentiviral vector had little effect on the virus titer compared with chicken β-globin insulator. Clonal analysis of transduced HL-60 cells revealed that ArsI protects the lentiviral vector from position effects regardless of its orientation. Furthermore, chromatin immunoprecipitation assays revealed that a high acetylation level was observed in the promoter of the insulated vector, whereas that of ArsI was independent of its anti-silencing capacity. In addition to it having little deteriorative effect on the virus titer, the identified anti-silencing effect of ArsI suggested its possibility for application in gene therapy.
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Keywords: *chromatin insulator; gene silencing; lentiviral vector; histone acetylation*

Introduction

Retroviral vectors have been extensively developed as vehicles for therapeutic genes. Although these vectors assure efficient gene delivery into host genome, expression from these vectors depends on the site of integration in the host genome.[1] This phenomenon, the so-called position effect variegation, is one of the major defects of these vectors. Another problem relevant to these vectors is the silencing of transgenes that had been highly transcriptionally active. This transgene silencing has been observed both *in vitro* and *in vivo* across various cell types and species,[2–5] and could be a significant threat to successful gene therapy since the correction of genetic disorders would require life-long expression of the therapeutic genes. The mechanism of transgene silencing remains to be elucidated; however, accumulating evidence suggest that epigenetic alterations of the transgene, such as DNA methylation and/or histone modifications, are likely components.[3,5,6] Engineering retroviral vectors to avoid epigenetic alterations is needed for successful application of gene therapy. Retroviral vectors currently in experimental and/or clinical practice are categorized into two groups: oncoretrovirus-based (eg Moloney-murine leukemia virus, MoMLV) and lentivirus-based (eg human immunodeficiency virus type-1, HIV-1) vectors. Lentiviral vectors have several advantages over oncoretrovirus vectors in that they have the ability to infect nondividing cells (eg neurons, myocytes and stem cells), which are expected targets for gene complementation.[7] Furthermore, lentiviral vectors possess a higher stability of expression *in vivo* than oncoretroviral ones, supporting their use for long-term corrections of genetic disorders.[8,9]

Chromatin insulator is a DNA sequence that serves as a boundary element between differentially regulated genes. Various insulators have been found from *Drosophila* and vertebrates, including chicken, mouse and human.[10–13] These insulators have two conserved properties,[14] one is an enhancer-blocking activity in which the insulator blocks enhancer/promoter interaction when placed in between them and the other is the protection from the position effect. Specifically, by flanking the promoter/reporter cassette with two insulators, the cassette will be isolated from the local chromosomal environment and therefore be protected from the position effect.

The most investigated vertebrate insulator, cHS4, is derived from the chicken β-globin gene locus control region.[2,10,15–19] Previous reports have shown that HS4 insulator protects transgenes from position effect variegation when introduced into MoMLV-based retroviral vectors.[20,21] Since the HS4 fragment is active in restricted cell types,[21] a quest for alternative insulators is necessary to propagate the use of the insulator in various cell types and tissues.

The 573 bp fragment of sea urchin arylsulfatase (Ars) gene locus (Ars insulator, ArsI) is reported to have typical features of an insulator such as blocking of enhancer/promoter interaction in sea urchin embryos

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and position effect protection in HeLa cells.[22] Since ArsI works in various cell types and across species, it has been suggested that this nonvertebrate-derived insulator might serve as a universal insulator working in various human tissues.[22–24] Therefore, we developed an HIV-1-based lentiviral vector carrying ArsI, and tested for its anti-silencing activity.

Results

Generation of insulator-containing lentiviral vectors

Previous studies have shown that transgenes flanked by two insulators were protected from position effects and/or silencing. Therefore, to flank the promoter/reporter cassette within the context of an HIV vector, we introduced insulator fragments into the lentiviral vector, pHR′, by replacing the EcoRV–EcoRV fragment of the 3′-U3 region with insulators (Figure 1a). By this strategy, the ArsI inserted should be copied to 5′-long terminal repeat (LTR) during reverse transcription and thereby ArsI in the 5′- and 3′-U3 regions would flank the whole reporter cassette, HygEGFP gene under the control of the cytomegalovirus (CMV) promoter. Amplification of the 5′-LTR by PCR shows that ArsI was successfully copied to the 5′-U3 region as expected (Figure 1b). Vector titers for HR/A+ and HR/A− were similar to the control vector, HR/cHG, indicating that ArsI does not interfere with lentiviral vector packaging and transduction (Table 1). Incorporation of cHS4 insulator into U3 region of LTR decreased the viral production, and the cHS4 sequence in 5′-LTR was partially deleted in the provirus (Figure 1b). Therefore, we could not test the ability of HS4 in the context of an HIV-based vector. To identify the process, in which HR/H+ and HR/H− vectors were truncated, we analyzed the integrity of the viral genome RNA in the packaging cells. We found that both of them harbored truncations (data not shown) in the viral genome RNA. This result suggests that cHS4 lost its integrity by an unexpected splicing in pHR′-based lentiviral vector.

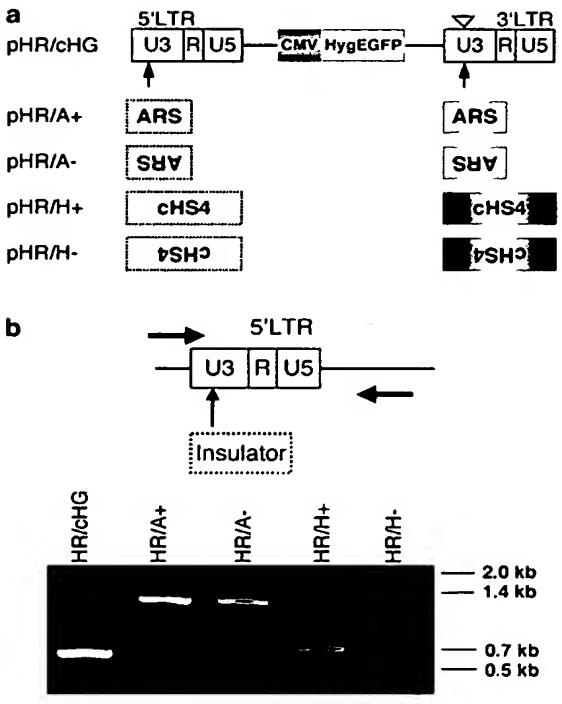

Figure 1 Construction of ArsI-containing lentiviral vectors. (a) Schematic diagram of lentiviral vectors. Top: control vector, pHR/cHG containing HygEGFP reporter driven by CMV immediate-early promoter and 79 bp deletion in the 3′-U3 region (triangle). Middle and bottom: vectors containing insulators in the 3′-U3 region of pHR/cHG. pHR/A+ carries ArsI in sense orientation of its native genomic locus, whereas pHR/A− carries antisense orientation. Dotted boxes illustrate ArsI copied into 5′-U3 region after integration and arrow indicates the insertion site of the insulators. (b) ArsI introduced into 3′-U3 was copied to 5′-U3 during the reverse transcription. Proviral sequences from lentivirally transduced HL-60 cells were amplified by PCR using primers specific for 5′-LTR. The expected size of the PCR product without insulators was approximately 0.7 kb, whereas the size with intact ArsI or cHS4 were 1.3 or 1.9 kb, respectively.

Table 1 Vector titers

Vector	*Titer (TU/ml)*
HR/cHG	1.5×10^5
HR/A+	1.1×10^5
HR/A−	1.1×10^5
HR/H+	3.9×10^4
HR/H−	6.6×10^4

Vector titers were determined according to EGFP expression levels of 293T cells transduced with serially diluted virus stocks.

Effect of ArsI on lentiviral vector silencing

To rapidly assess the antisilencing capacity of insulator-containing vectors, it was our intention to use CMV promoter, which is highly susceptible to silencing. For silencing protection assay, we introduced lentiviral vectors into various cell lines including HL-60, Huh-7, K562, MCF-7, MT-2, 293 T and CCE cells. Cells were transduced with lentiviral vectors, HR/cHG, HR/A+ or HR/A− at a multiplicity of infection (MOI) of 1, and were selected for hygromycin-resistant cells. Primary transduction efficiency varied among cell types from approximately 3% in HL-60 cells to 90% in 293 T cells. After we confirmed that almost all cells were positive for expression of EGFP, such cells were selected and cultured with hygromycin for an additional 2 weeks to eliminate untransduced cells. Thereafter, cells were cultured without hygromycin and periodically tested for EGFP expression. Long-term silencing was observed in HL-60, Huh-7, K562, MCF-7 and CCE cells, but not in MT-2 and 293 T cells. Extinction patterns differed among cell lines (Figures 2 and 3).

In HL-60 cells, EGFP expression from HR/cHG and HR/A+ started to diminish soon after the removal of hygromycin, and the rates of EGFP-positive cells and mean fluorescence intensity (MFI) values declined to approximately 30% of their hygromycin-selected counterparts (Figure 2b and c). On the other hand, EGFP expression from HR/A− was maintained throughout the assay period (Figure 2a–c). Similar results were obtained by three independent experiments. This orientation-specific activity of ArsI was consistent with a previous report in which only the antisense orientation blocked the enhancer–promoter interaction in sea urchin and

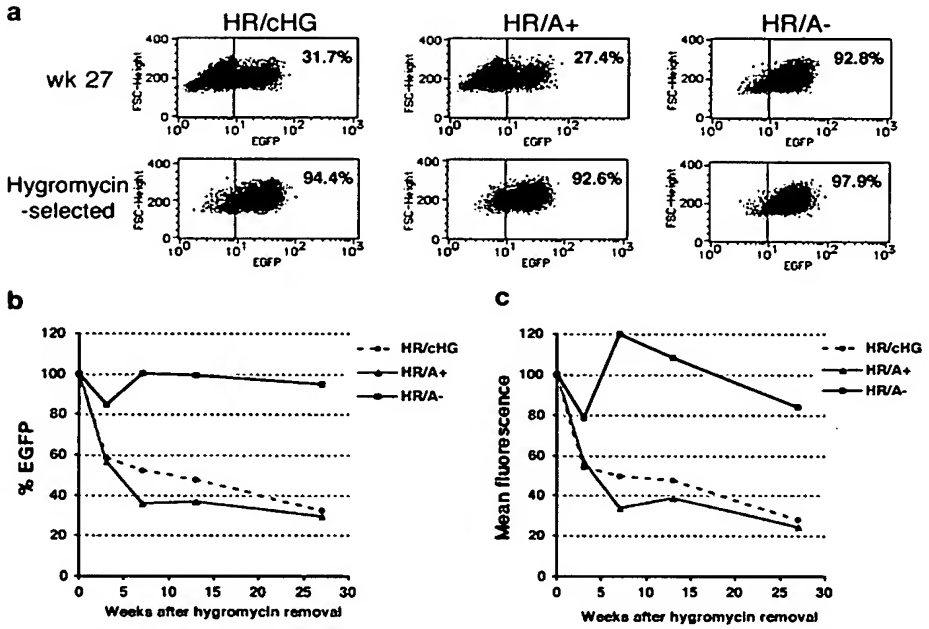

Figure 2 ArsI prevented silencing of lentiviral vectors in HL-60 cells. (a) Dot plots of FACS analysis for EGFP expression. Transduced HL-60 cells were selected by hygromycin for 4 weeks, and then cultured without hygromycin. Dot plots show data from the 27th week after hygromycin removal (top) and cells cultured in the absence of hygromycin (bottom). Sequential changes of EGFP expression after the removal of hygromycin are shown by the percentage of EGFP-positive cells (b) and by MFI (c).

Table 2 Proviral DNA copy number

	Relative copy no.	
	Hygromycin selected	*Week 20*
HR/cHG	1.000	0.993
HR/A+	1.000	0.871
HR/A−	1.000	0.884

Relative copy no. for transduced HL-60 cells. Values for hygromycin-free weeks 20 cells are expressed as the values relative to their hygromycin-selected counterparts.

Table 3 Variations of vector copy number in individual clones

	Clone no.						
	1	*2*	*3*	*4*	*5*	*Mean*	*s.d.*
HR/cHG	1.000	1.507	2.322	1.240	1.037	1.421	0.542
HR/A−	1.019	1.150	1.263	1.055	1.237	1.145	0.108

Values are expressed as the amount of vector DNA relative to HR/cHG clone no. 1.

protected the transgene from position effect in HeLa cells.[22]

To exclude the involvement of gene loss in the apparent extinction of expression, we performed real-time PCR with the genomic DNA from the transduced HL-60 cells. In the cells harvested 20 weeks after hygromycin removal, there were no significant differences in proviral copy number compared with the hygromycin-selected counterparts (Table 2). We also quantified the amount of proviral sequence in the HL-60 clones isolated after gene silencing took place. There were no significant differences in copy number either among clones or vector types (Table 3).

Neither orientation of ArsI could prevent lentiviral vector silencing in K562, Huh-7 and MCF-7 cells (Figure 3a–c). We also attempted to transduce CCE cells; however, the expression of EGFP from pHR′-based vectors was trace and could not be determined by fluorescence-activated cell sorting (FACS) analysis. Therefore, we used the alternative vector, pCSII, the expression of which is strengthened by the inclusion of woodchuck hepatitis virus post-transcriptional regulatory element (WPRE). Use of pCSII enabled the detection of EGFP expression by FACS. Moderate protection of silencing was observed in CCE cells when CSII/A− was introduced (Figure 3d and e).

Taken together, these results show that ArsI protects lentiviral vector from silencing in an orientation- and cell type-dependent manner.

Comparison of ArsI and cHS4 in their antisilencing capacity

To compare ArsI and cHS4 in their anti-silencing capacity, we utilized an MoMLV-based vector and inserted insulator fragments into 3′-U3. Both ArsI and cHS4 were copied to 5′-U3 and remained intact after integration (data not shown). RCV vectors with or without insulators were introduced into HL-60 cells and their EGFP expressions traced. EGFP expression from the control vector without insulator, RCV/HG, extinguished within the assay period rather faster than with the HIV-based vector (Figures 2 and 4). RCV/A+ was also silenced while RCV/A− was protected from silencing (Figure 4). Similar orientation dependency was also observed in cHS4-containing vectors and their

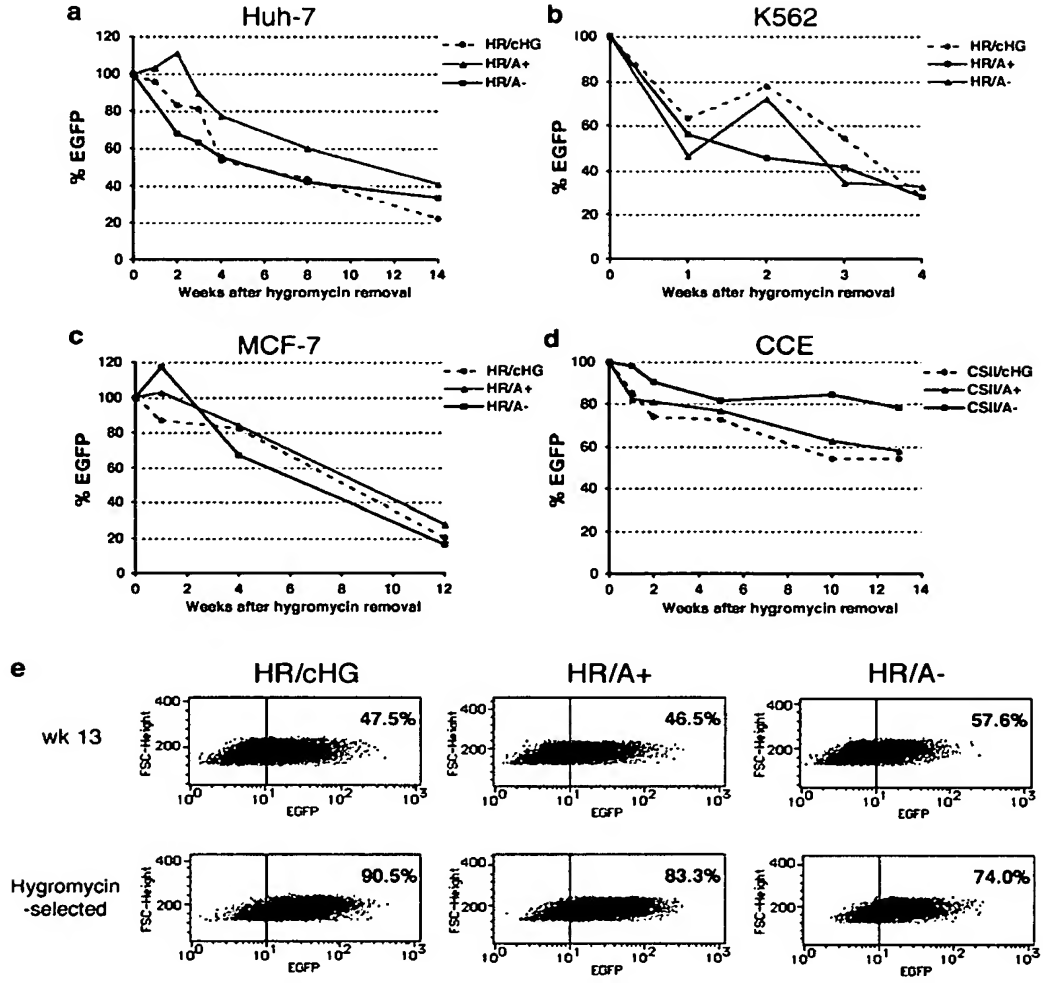

Figure 3 Silencing protection ability of ArsI in various cell lines. Sequential changes of EGFP expression of transduced (a) Huh-7, (b) MCF-7, (c) K562 and (d) CCE cells are shown. (e) Dot plots of EGFP expression in CCE cells at week 13. See Figure 2 for details.

anti-silencing capability of cHS4 was equivalent to that of ArsI in HL-60 cells.

Effect of ARS insulator on position effect variegation in HL-60 cells

We next tested the effect of ArsI on position effect variegation, since the assurance of position independent expression by an insulator may play a role in protection against silencing. HL-60 cells were transduced with HR/cHG, HR/A+ or HR/A−, and selected for hygromycin-resistant cells and subsequently cloned by limiting dilution. 10 clones were selected from each cell pool and tested for EGFP expression. Clones were assigned to three classes (high, medium and low) according to their MFI values. Data from three independent experiments are summarized in Figure 5. Expressions of HR/cHG clones varied and were equally distributed to all classes. Surprisingly, both HR/A+ and HR/A− clones showed unified expression. In both vectors, low-class clones were eliminated, which means that negative chromosomal effects were declined by the incorporation of ArsI. These results indicate that ArsI protects lentiviral vectors from position effect variegation regardless of the orientation, suggesting that this activity was independent of silencing protection.

Effects of insulators on differentiation-associated silencing in HL-60 cells

Transgene silencing after differentiation is one of the major obstacles to a successful gene therapy application.[25,26] Therefore, we studied whether ArsI can protect lentiviral vectors from differentiation-associated silencing. As a differentiation model, we induced granulocytic differentiation of HL-60 cells by dimethylsulfoxide (DMSO) treatment. Hygromycin-resistant transduced cell pools were induced to differentiate by DMSO treatment for 6 days and were then tested for EGFP expression and nitro blue tetrazolium (NBT) reduction capacity, which reflects the granulocytic phenotype. Positivities of NBT staining were at comparable levels for all three transduced cells, ranging from 73 to 78% of total (Figure 6, left). EGFP expression of induced HR/cHG cells was reduced to average of 42.8% relative to an uninduced counterpart (Figure 6, right). Similarly, EGFP

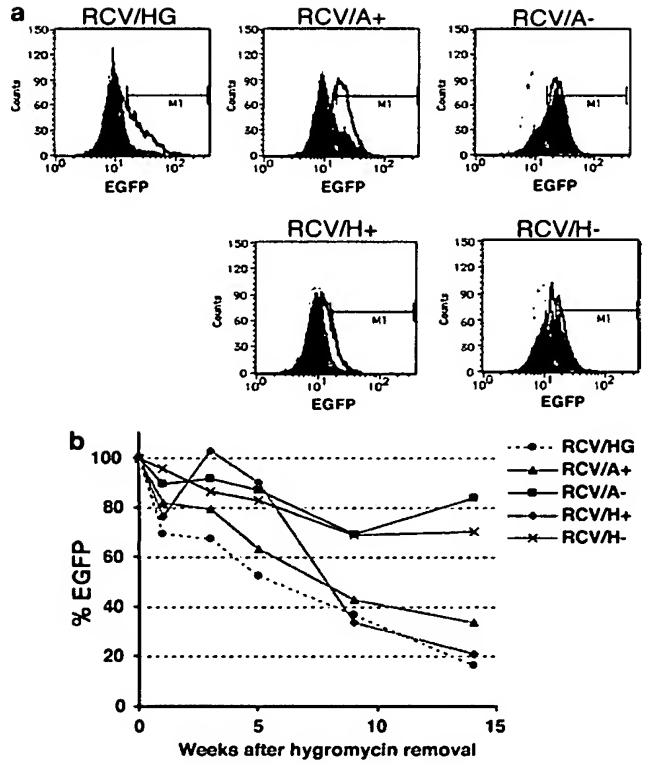

Figure 4 Comparison of antisilencing abilities between ArsI and cHS4. HL-60 cells were transduced with MoMLV-based vector, RCV, containing ArsI or cHS4, and then EGFP expressions were monitored periodically. (a) Histograms of EGFP expression 14 weeks after hygromycin removal (dotted line: untransduced; solid line: cells cultured under hygromycin selection; filled histogram: cells cultured in the absence of hygromycin) (b) Time course of EGFP-positive cells after the removal of hygromycin. See Figure 2 for details.

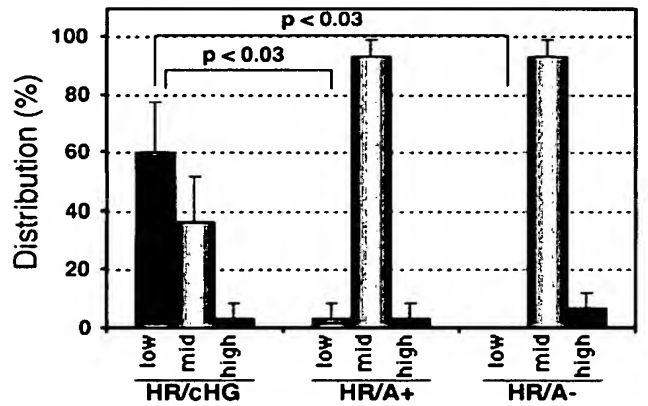

Figure 5 Clonal analysis of transduced HL-60 cells. Clones were selected by limiting dilution of hygromycin-resistant cell pools and were maintained in the presence of hygromycin. Clones were classified into three groups (low, mid and high) according to their MFI of EGFP. Values are means ± s.d. of three independent experiments.

expression of induced HR/A+ and HR/A− cells declined to 33.7 and 33.5%, respectively (Figure 6, right). These results suggested that ArsI did not protect lentiviral vector from silencing after granulocytic differentiation of HL-60 cells.

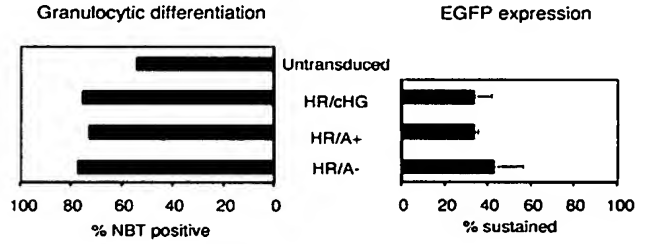

Figure 6 ArsI does not prevent differentiation-associated silencing in HL-60 cells. Stably transduced HL-60 cells were induced to differentiate into granulocytes by 1.25% DMSO treatment for 6 days. Cells were tested for NBT staining (left) as well as EGFP expression (right). EGFP expression level was calculated as the percentage of cells remaining positive after differentiation. Values are means ± s.d. of three independent experiments.

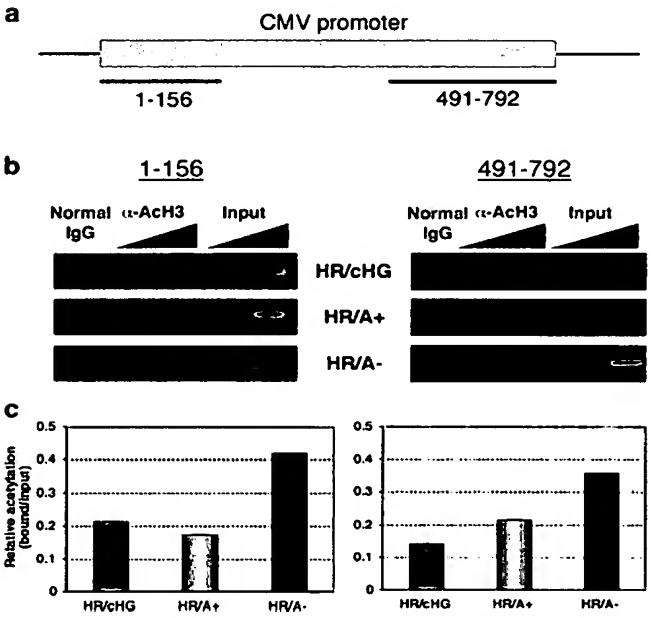

Figure 7 Histone acetylation status of CMV promoters from transduced HL-60 cells. In vivo crosslinked chromatin samples from transduced HL-60 cells were subjected to ChIP assays using anti-acetylated histone H3 antiserum. (a) Diagram of the CMV promoter sequences targeted for PCR analyses. Regions corresponding to nucleotide nos. 1–156 and 491–792 of CMV promoter were chosen as targets. (b) PCR detection of acetylated histone-associated sequence within two separate regions of the CMV promoter. (c) Quantification of relative acetylation levels. Values were calculated as relative signal intensity normalized by input DNA.

Effect of ArsI on histone acetylation

To clarify the mechanism of silencing protection by ArsI, we focused on the chromatin structure within the vector provirus, since transgene silencing is often regarded as a direct consequence of the inactivation of the local chromatin structure.[2,5,6] Especially, acetylation status of lysine residues within histone tails are generally accepted as markers of chromatin activation.[27] For this purpose, we performed ChIP assay to detect histone acetylation levels of proviral ArsI and CMV promoter (Figure 7). As shown, insulated HR/A− exhibited a high histone H3 acetylation level in the CMV promoter region compared with silenced HR/cHG and HR/A+

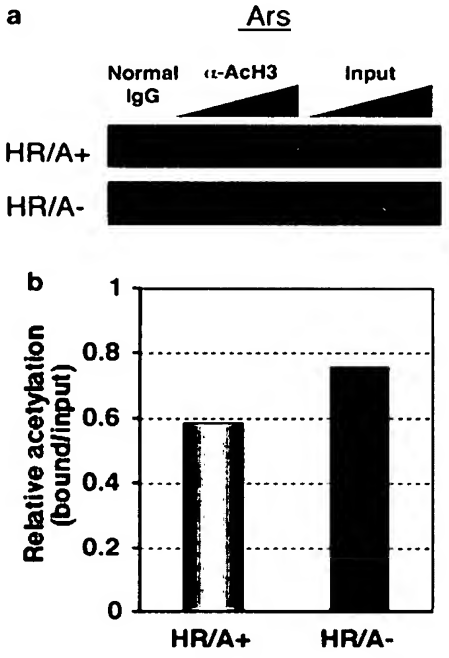

Figure 8 Histone acetylation status of ArsI from transduced HL-60 cells. (a) PCR detection of acetylated ArsI. (b) Quantification of relative acetylation level. See Figure 7 for details.

(Figure 7b and c), which was consistent with the notion that the histone acetylation level at a promoter positively correlates with transcriptional activity. When we compared the level of H3 acetylation at proviral ArsI from silenced HR/A + and nonsilenced HR/A−, the levels of acetylation were similar, indicating that the chromatin structure of ArsI was not responsible for silencing protection (Figure 8). However, ArsI− could inhibit the silencing in an orientation-dependent manner, suggesting a mechanism other than histone acetylation.

Effect of ArsI on DNA methylation

DNA methylation is a key mediator of transgene silencing that links with histone acetylation.[2,5,28] To clarify the involvement of CpG methylation in lentiviral silencing, we analyzed the methylation of CpG sites in the CMV promoter by bisulfite sequencing method. We analyzed 10 CpG sites close to the 3′-end of the CMV promoter from the silenced HR/cHG provirus. In eight CpG sites, there was no methylation at all, and two sites were partially methylated (10% in each site: data not shown). This result indicates that promoter DNA methylation was not involved in lentiviral silencing, and therefore, silencing protection by ArsI was not a consequence of the inhibition of DNA methylation.

Discussion

Gene delivery systems based on retroviral and lentiviral vector have been extensively developed, and the adaptation of these vectors for gene therapy is within reach.[29] However, there are some impediments to be overcome before their successful use. One is the attenuation of the transgene expression over time caused by epigenetic alterations such as DNA methylation and histone tail modifications.[5] To overcome this obstacle, use of epigenetic regulatory elements such as insulators, matrix attachment regions and locus control regions have been attempted.[20,21,30–33] In this study, we found that sea urchin ArsI protects an HIV-based vector from long-term silencing in cell type- and orientation-dependent manner.

ArsI was effective in HL-60 and CCE cells, but not in other cell lines. This cell type restriction may be attributed to the existence of a transacting factor interacting with ArsI, the expression of which differs among cell types or maturation stages. However, well-known insulator-binding protein CTCF is not a likely candidate since this protein is reported to be strongly associated with enhancer-blocking activity, rather than anti-silencing effect in studies using cHS4.[18] One can also attribute the cell type selectivity to the physiological role of insulators, that is, the regulation of stage-specific gene expression by regulating higher chromatin structure.[16,17] Recently, double-stranded DNA-binding protein, SATB1, was reported to play a key role in forming thymocyte-specific nuclear architecture.[34] SATB1 is proposed to be involved in the chromatin loop formation and regulation of histone modifications.[34,35] Such factors with similar properties may contribute to the ArsI function and other insulators in general. Furthermore, ArsI possessed anti-silencing activity in CCE, murine ES cells, contrary to the disability of cHS4 in murine and human ES cells reported previously.[21,36] These observations indicate that the way of cell type selection differ among insulators.

We also observed an orientation dependency of the anti-silencing effect of ArsI. It has been suggested that ArsI exerts its enhancer-blocking activity against downstream enhancer in the native chromosome locus.[22] Thus, ArsI may substantially function in a directional manner both in enhancer-blocking activity and in protection against silencing. Directional action by an insulator has previously been reported for the insulator derived from *Drosophila* gypsy retrotranspozon.[3] The gypsy insulator affects chromatin structure directionally, and this may also be the case for ArsI.

It has generally been considered that long-term silencing protection is likely to be a consequence of position effect protection.[37] However, a recent study by Recillas-Targa *et al*[18] indicated that different domains of cHS4 are responsible for position effect and long-term silencing protections. In our study, ArsI in a plus orientation prevented variability of expression, while it failed to maintain expression in the long term. Taken together, these observations indicate that different mechanisms are involved in primary integration site-dependent expression and the subsequent transgene extinction. Furthermore, ArsI seems to have the potential to protect both of them.

All experiments in our study were performed after drug selection for the cells expressing the transgene. This fact poses a marked difference between our study and others that reported the anti-silencing activity of insulators and other cis-elements.[20,21,38,39] While others have analyzed the proviruses in unfavorable integration sites, we have precluded them by hygromycin selection prior to the analyses. Thus, we have monitored the gradual inactivation of the transgenes, whereas previous reports have observed the expression of the transgenes integrated in inactive loci. A plausible interpretation of our results is that ArsI assures the long-term expression of

the transgene with a transcriptional activity higher than a certain threshold.

In our study, cHS4 in either orientation was deleted in the context of lentiviral vector, while ArsI remained intact. RT-PCR experiment revealed that truncation had already taken place at the viral packaging step. This result suggests that lentiviral vector containing cHS4 was susceptible to unexpected splicing during the synthesis of the viral genome RNA. This finding is not consistent with the previous report, in which cHS4 remained intact throughout the lentiviral vector transduction.[38] This discrepancy may be due to the difference in the vector background and/or difference in the site of cHS4 insertion. Indeed, in our experiment using MoMLV-based vector, cHS4 fragment was not altered, supporting the idea that vector background affects the splicing pattern.

Gene silencing is closely related to the epigenetic regulation of the local chromosomal environment such as DNA methylation and histone modifications.[40] In addition, insulators have been shown to exert their activity through the regulation of the chromatin structure.[3,16,17] Therefore, we focused on the epigenetic changes of the integrated vector sequence to unravel the mechanism of action of ArsI. We analyzed the acetylation status of histone H3 in the provirus, which reflects the open state of chromatin fiber as well as DNA methylation. We found that DNA methylation was not responsible for lentiviral silencing, and that insulated promoter had higher level of histone acetylation than noninsulated ones. This finding suggests that ArsI assured the sustained lentiviral expression by maintaining active chromatin structure in the absence of DNA methylation. The level of histone acetylation is controlled by the balance between histone acetylases (HATs) and deacetylases (HDACs) activities, suggesting that ArsI function involves the recruitment of HATs and/or rejection of HDACs.[41]

Since retrovirus vectors integrate into host chromosomes, the risk of oncogenesis caused by insertional mutagenesis is a concern. Recently, such phenomena were actually observed in a mouse model and in human clinical practice.[42,43] In both cases, oncogenesis seems to be a consequence of the activation of an oncogene adjacent to the integrated provirus. Since insulators serve as boundary elements in chromosomes, they might prevent such unexpected activations of adjacent genes and oncogenesis.

Inclusion of ArsI into lentiviral vectors would be advantageous for various applications, since it remains intact after integration into the host genome and does not affect vector titer. To confirm its potential in gene transfer techniques, *in vivo* studies using model animals need to be performed.

Materials and methods

Vector construction

Lentiviral vectors were sourced from an HIV-1-based vector, pHR′ (generous gift from Inder Verma, Salk Institute, La Jolla, CA, USA).[7] First, central polypurine tract (cPPT) from HIV-1 genome was introduced into the vector to increase transduction efficiency.[44] cPPT was amplified using an HIV-1 molecular clone, pNL4-3, as a substrate with primers as follows: cPPT-F, 5′-GCAGA-TCTAC-ATATG-GCAGT-ATTCA-TCC-3′; cPPT-R, 5′-TAGGA-TCCCC-AAACT-GGATC-TCTGC-TGTCC-3′. After *Bgl*II/*Bam*HI digestion, amplified cPPT was inserted into the *Bam*HI site of pHR′. To engineer the U3 region of the 3′-LTR of pHR′, a 2.2 kb *Kpn*I–*Xba*I fragment containing the U3 region of 3′-LTR was subcloned into the corresponding site of pUC119 to generate pUC/3′-LTR. ArsI was digested from pBluescript containing ArsI in its *Sma*I site with *Sma*I and *Eco*RI, and then blunted and ligated into *Eco*RV-digested pUC/3′-LTR. Although digestion with *Eco*RV creates a 79 bp deletion in the U3 region, the deletion of this sequence does not affect the ability of the vector.[45] A *Kpn*I–*Xba*I fragment from pUC/3′-LTR-containing ArsI in sense and antisense orientations was returned into pHR′ to generate pHR/A+ and pHR/A−, respectively. Simultaneously, a *Kpn*I–*Xba*I fragment from the self-ligated vector was inserted to pHR′ to generate the control vector, pHR/cHG. Vectors containing cHS4 insulators (pHR/H+ and pHR/H−) were constructed as well as pHR/A. A cHS4 fragment was amplified from chicken genomic DNA by PCR using the following primers: HS4-F, 5′-GAGCT-CACGG-GGACA-GCC-3′; HS4-R, 5′-AATAT-TCTCA-CTGAC-TCCGT-CCT-3′. After cloning the 1210 bps PCR fragment, 96% homology to the reported cHS4 sequence was confirmed. HygEGFP, a fused gene between the hygromycin resistance and enhanced green fluorescence protein genes, was used as a reporter gene, and was transcribed by cytomegalovirus immediate-early promoter (CMV). *Xho*I and *Kpn*I sites were added to the 5′- and 3′-end of CMVie, respectively, using the following primers: CMV-F, 5′-AGC-CTCGAG-TCAAT-ATTGG-CCATT-AGCCA-3′; CMV-R, 5′-AC-GGTACC-CTGAC-TGCGT-TAGCA-ATTTA-3′. Amplified product was ligated to *Xho*I—*Kpn*I-digested pHygEGFP (Clontech), the plasmid containing HygEGFP gene. The CMVie/HygEGFP cassette was obtained by *Xho*I–*Sal*I digestion and was inserted into the *Xho*I site of pHR′-based vectors.

For lentiviral transduction of CCE cells, pCSII-CMV-MCS (generous gift from Hiroyuki Miyoshi, RIKEN, Tsukuba, Japan) was used.[46,47] This vector contains WPRE, which has been reported to enhance retroviral expression.[48] The HygEGFP gene was excised from pHygEGFP by *Xho*I/*Not*I digestion and inserted into the corresponding site of pCSII-CMV-MCS to generate pCSII/cHG. Blunted ArsI was inserted into *Eco*RV-digested pCSII/cHG in both directions to generate pCSII/A+ and pCSII/A−.

The MoMLV virus-based vector, pRCV, was established by replacing the neomycin phosphotransferase gene and the SV40 promoter of pLNSX with a multicloning site-containing oligonucleotide. The *Sal*I/*Xho*I-digested HygEGFP gene was inserted into the corresponding site of pRCV to generate the control vector, pRCV/HG. To manipulate 3′-LTR of pRCV, a *Bgl*II/*Ase*I-digested 3′-LTR-containing fragment was subcloned into pIRES2-EGFP (pIRES2-EGFP-LTR). *Nhe*I-digested pIRES2-EGFP-LTR was blunted and then blunted insulators, ArsI or cHS4 were inserted in either orientation to produce pRCV/A+, pRCV/A−, pRCV/H+ and pRCV/H−, respectively.

Production of recombinant virus and titration

Lentiviral vectors were produced by transient transfection of a human embryonal kidney cell line, 293 T, as described elsewhere with minor modifications.[49,50] Briefly, 293 T cells were inoculated onto a six-well plate,

and 24 h later were transfected with three plasmids using Fugene reagent (Roche diagnostics). The composition of the three plasmids is as follows: 3 μg of transfer vector, 3 μg of pCMVΔ8/9, which encodes HIV-1 viral proteins, and 2 μg of pcDNA-VSV-G (generous gift from Hiroyuki Miyoshi, RIKEN, Tsukuba, Japan), which encodes vesicular stomatitis virus envelope glycoprotein. At 48 h after transfection, virus-containing supernatants were collected and filtrated through 0.45 μm cellulose acetate filter, and stocked at −80°C until use.

Similarly, RCV-based vectors were produced by cotransfection of equal amount of pRCV plasmids and pcDNA-VSV-G into gag-pol-expressing packaging cells. Culture supernatant was collected, aliquoted into small fractions and stocked at −80°C until use.

Serially diluted virus stocks were applied for the determination of vector titer. For the titration, 293T cells were challenged with serially diluted virus solutions for 16 h in the presence of 4 μg/ml polybrene. After 48 h, cells were harvested, fixed with 0.25% paraformaldehyde and analyzed for EGFP expression by FACS. FACS was performed using a FACSCAN flowcytometer (Becton Dickinson) and analysis using CELLQUEST software. Virus titers were calculated as transducing units per ml.

Cell lines

A human myeloid cell line, HL-60, and an erythroid cell line, K562, were grown in RPMI 1640 medium (Sigma) supplemented with 10% fetal calf serum (FCS) and 3% L-glutamine. 293 T cells and a human breast cancer cell line, MCF-7, were grown in Dulbecco's modified Eagle's medium (DMEM) (Sigma) supplemented with 10% FCS and 3% L-glutamine. A human hepatocellular carcinoma cell line, Huh-7, was grown in DMEM supplemented with 10% FCS, 3% L-glutamine and a nonessential amino-acid solution. A murine embryonic stem cell line, CCE, was grown on gelatin-coated plates and maintained in knockout DMEM (Invitrogen) supplemented with 15% FCS, 100 nM β-mercaptoethanol, nonessential amino-acid solution, 3% L-glutamine and 5000 U/ml recombinant leukemia inhibitory factor (Chemicon).

Transduction and silencing protection assay

Transduction of the lentiviral vector was performed for 16 h using virus solutions containing 4 μg/ml polybrene. HL-60, Huh-7, K562, MCF-7 and CCE cells were transduced with lentiviral vectors at a MOI of 1. After 48 h culture, optimal doses of hygromycin B (Sigma) were added for positive selection. Dosages of hygromycin used were as follows: HL-60, 0.75 mg/ml; Huh-7 and K562, 0.4 mg/ml. Hygromycin selection was extended for 4 weeks to ensure the clearance of untransduced cells. Subsequently, hygromycin was removed and EGFP expression was traced periodically throughout the assay period. The same transduced cells were cultured with hygromycin as a positive control for EGFP expression.

PCR analysis of proviral DNA

Duplication of insulator fragment in 5′-U3 was confirmed by PCR amplification using a 5′-LTR-specific primer pair. HL-60 cells were transduced with HR/cHG, HR/A+ or HR/A−, and hygromycin-resistant cells were selected. Cells were harvested and genomic DNA was extracted using DNAzol reagent (Invitrogen). Approximately 0.5 μg DNA was used to amplify proviral 5′-LTR using *rTaq* polymerase (TOYOBO). Primers used were as follows: HXB2/U3-F, 5′-AAGGG-CTAAT-TCACT-CCCAA-3′; HXB2/psi-R, 5′-TG-CGTCG-AGAGA-GCTCT-GGTTT-3′.

Isolation of HL-60 clones transduced by recombinant viruses

Individual transduced HL-60 clones were obtained by limiting dilution of the hygromycin-resistant cell pool. Part of the hygromycin-resistant cells obtained in the silencing protection assay were inoculated onto a 96-well plate at a density of 0.5 cells/well and cultured in the presence of hygromycin. After recovering adequate cell numbers, cells were harvested and tested for EGFP expression by FACS. Clones were classified into three groups (low, medium and high) according to their MFI of EGFP.

Granulocytic differentiation of HL-60 cells

Induction of HL-60 cells into granulocyte lineage was performed by DMSO treatment as described previously.[51] Hygromycin-resistant cell pools were treated with 1.25% DMSO for 6 days. In this induction period, hygromycin was removed to keep silenced cells viable. Parallel control cultures were maintained without DMSO and hygromycin. The level of granulocytic differentiation was measured by NBT reduction assay. Cells before and after induction were tested for EGFP expression. MFI values of induced cells were divided by the MFI values of uninduced cells to formulate relative MFIs.

Chromatin immunoprecipitation (ChIP) assays

ChIP assays were performed as described previously.[52] Briefly, HL-60 cells (5×10^5 cells/antibody) were fixed with formaldehyde and then sonicated to obtain soluble chromatin. Soluble chromatin was immunoprecipitated with anti-acetylated histone H3 antibody (Upstate Biotechnology) overnight at 4°C. Immunoprecipitates were collected with 50% protein A and G-Sepharose slurry preabsorbed with 0.1 mg/ml sonicated salmon sperm DNA. Consequently, purified DNA was subjected to PCR reactions using primer sets specific for CMV promoter or ArsI. Primers used were as follows: CMV 1F, 5′-GAGCA-GCATC-TCGAG-TCAAT-ATTG-3′; CMV 156R, 5′-CTAGT-CAATA-ATCAA-TGCCA-ACATG-3′; CMV 491F, TACGT-ATTAG-TCATC-GCTAT-TACC-3′; CMV 792R, 5′-ATCCT-CTAGA-GGTAC-CCTGA-CTGCG-T-3′; Ars F, 5′-AGCAG-AACCC-CTGTA-AGCTC-AG-3′; Ars R, 5′-GCTGA-AATTA-CATAA-CTTCC-CGAGA-T-3′. PCR products were separated on agarose gel and the results were quantified using ATTO densitometry software. Values were calculated as mean signal intensity of serially diluted samples normalized by input DNA.

Real-time PCR

Genomic DNA from lentivirally transduced HL-60 cells was analyzed by real-time PCR using ABI Prism 7700 sequence detector (Applied Biosystems) with TaqMan Universal Master Mix. CMV promoter as well as internal control, recombination activated gene-1 (RAG-1), were quantified using the following primers and TaqMan probes: CMV 1F, CMV 156R (see above), CMV probe,

5′-TGGCC-ATTGC-ATACG-TTGTA-TCTAT-ATCAT-AA-3′, RAG-1 F; 5′-CCCAC-CTTGG-GACTC-AGTTC-T-3′, RAG-1 R; 5′-CACCC-GGAAC-AGCTT-AAATT-TC-3′; and RAG-1 probe, 5′-CCCCA-GATGA-AATTC-AGCAC-CCACA-TA-3′. Amount of proviral sequence in hygromycin-free culture appears as the value relative to hygromycin-selected counterpart normalized by RAG-1 gene. Values for cloned HL-60 cells were shown as the value relative to a representative clone, which was designated as 1.

Bisulfite genomic sequencing

Sodium bisulfite treatment of genomic DNA was performed as described previously.[53] 178 bp fragment of CMV promoter region was amplified using bisulfite-treated genomic DNA extracted from lentivirally transduced cells by nested PCR. Primers used in the first PCR were: CMVSB571-F, GGGAT-TTTTA-AGTTT-TTAT-3′; CMVSB782-R, 5′-CAATT-TAACT-ATAAT-AAACT-ACC-3′. Second primers were: CMVSB605-F, 5′-GGGAG-TTTGT-TTTGG-TATTA-3′; CMVSB758-R, CAATA-AAACT-TCTAA-TAATC-3′. PCR products were cloned into pGEM-T easy plasmid (Promega), and 10 clones were randomly selected for sequence determination using ABI 310 autosequencer (Applied Biosystems).

RT-PCR

Total RNA of the packaging cells was extracted using Trizol reagent (Invitrogen). RT-PCR was performed using RNA LA PCR Kit Ver. 1.1 (Takara Bio Inc.) following the manufacturer's protocol. Primers used for the amplification of proviral cDNA are: HRKpn-F; 5′-GTCAC-ACCTC-AGGTA-CCTTT-AAG-3′, HRU3-R; 5′-CAAGC-GGGTG-TTCTC-TCCTT-CATTG-3′.

Statistical analysis

Statistical analyses were performed by Welch's *T*-test.

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